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VRK3-mediated inactivation of ERK signaling in adult and embryonic rodent tissues

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Abstract

Vaccinia-related kinase 3 (VRK3), previously characterized as a direct activator of vaccinia H1-related (VHR) phosphatase, inactivates extracellular signal-regulated kinase (ERK) in the nucleus of neuronal cells. Here we show that VRK3 is expressed in various other rodent tissues and in embryos, and regulates VHR phosphatase activity in these tissues. We observed colocalization of VRK3 and VHR in the testis tissue and could detect protein complex containing VRK3, VHR and ERK in immunoprecipitation analysis. Notably, the addition of recombinant VRK3 protein to total protein lysates, obtained either from adult tissues or embryos, enhanced the phosphatase activity of VHR, but not the activity of MKP3. The results further indicate that the VHR–VRK3 complex is a phosphatase-active form. In addition, we found that VRK3 can regulate EGF-induced cellular growth signaling that is mediated by ERK activation. Our results suggest that in addition to neuronal cells, various other rodent adult tissues and embryos possess a common signaling mechanism which is involved in an indirect regulation of ERK activity by VRK3-mediated VHR activity.

Keywords: Vaccinia-related kinase 3; Vaccinia H1-related phosphatase; Extracellular signal-regulated kinase; Mitogen-activated protein kinase phosphatase

1. Introduction

There are three members of the vaccinia-related kinase (VRK) Ser/Thr protein kinase family in mammals: VRK1, VRK2 and VRK3 [1,2]. VRK1 participates in the regulation of the cell cycle progression, particularly during mitosis, and its *Drosophila* homolog (known as nucleosomal histone kinase 1 (NHK1)), functions as a histone kinase in this process [3]. Recently we characterized the cellular function of VRK1 as a mitotic histone kinase. We demonstrated that VRK1 is involved in chromatin condensation by directly phosphorylating the histone H3 at its Thr3 and Ser10 residues during G2- to M-phase transition [4]. VRK1 is also known to regulate nuclear envelope assembly during mitosis by regulating the phosphorylation status of the barrier-to-autointegration factor (BAF) [5]. In addition, VRK1 is known to phosphorylate novel regulatory residues, and therefore increases the stability and activity of several transcriptional factors including p53 [6,7], ATF2 [8] and cJun [9].

Studies examining the role of various human splice variants of human VRK2, including VRK2A and VRK2B, demonstrate that VRK2A is found in the cytoplasm surrounding the endoplasmic reticulum and mitochondria, whereas VRK2B localizes not only to the nucleus but also to the cytoplasm [10]. These studies found that VRK2A is expressed in all cell types tested, whereas VRK2B is expressed in cell lines in which VRK1 is localized to the cytoplasm [10]. In the nucleus, VRK2B can phosphorylate tumor suppressor protein p53 at Thr18, suggesting that it may complement the activity of VRK1 [10]. Given the high amino acid sequence identity in the kinase domain of VRK1 and -2, they are believed to have some common substrates such as BAF [5] and p53 [10]. In contrast, both the kinase domain and the non-kinase regulatory domain of VRK3 are quite different from those of VRK1 and -2 [1]. Although vaccinia H1-related (VHR) phosphatase, an atypical mitogen-activated protein kinase (MAPK) phosphatase (MKP), is known to interact with VRK3 [11], no known VRK3 substrates have been identified to date.

The duration of activation and the subcellular localization of ERK pathway must be tightly controlled. This is important in order to guarantee the fidelity of integrated biological responses

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and the signal transduction processes required for the control of proliferation, differentiation and development [12,13]. Upon activation, ERK translocates to the nucleus and induces the expression of genes required for unique physiological processes by phosphorylating stimuli-specific transcription factors [14,15]. In contrast, MKPs negatively regulate MAPK activity by dephosphorylating critical phospho-residues required for the MAPK activation, and play an important role in determining the magnitude and duration of MAPK activity [16]. A dozen MKPs have been identified to date, and are distinguished by differences in their MAPK specificity and subcellular localization [17,18]. Based on their expression profiles, MKPs are largely categorized into two groups: inducible or constitutive. The genes of inducible MKPs, such as MKP1 and MKP2, are immediately expressed upon MAPKs activation, forming a negative feedback loop. After transient expression, they are rapidly degraded in a proteasome-dependent manner *via* MAPK-induced phosphorylation of MKP [19,20]. In contrast, constitutively expressed MKPs, such as VHR [11,21,22] and MKP3 [23], are responsible for the maintenance of MAPKs activity in quiescent cells as well as stimulated cells. The enzymatic properties of MKP3 and VHR seem somewhat different. MKP3 generally exists in an active state [24], whereas VHR remains rather inactive in resting cells, or at least in neuronal cell lines such as PC12 and HT22 cells, as well as in the Jurkat T immune cell line [11,22]. Recently we disclosed the molecular mechanism by which VRK3 modulates VHR activation [11]. Upon stimulation of the ERK pathway, the transcription of VRK3 is induced. In terms of a negative feedback system, we found that ERK activity was downregulated by VRK3-mediated VHR phosphatase activation which specifically targets phospho-ERK [11]. In T-cells, an alternative VHR activation mode is accomplished by a protein tyrosine kinase called ZAP70 that is involved in T-cell receptor activation, *via* Tyr138 phosphorylation of VHR [22]. Meanwhile, a recent study has revealed a role for VHR in cell cycle progression. During the cell cycle, VHR protein expression varies such that its expression peaks in the mitotic phase compared to the G1 phase [25]. Importantly, depletion of VHR expression using RNA interference results in sustained activation of ERK and JNK, causing cell cycle arrest at the G1/S or G2/M transitions, and also induces cellular senescence [25].

In the present study, we provide evidence that VRK3 regulates VHR activity in various tissues obtained from adult and embryo rodents. Our results demonstrate that VRK3 is a direct activator of VHR and negatively regulates ERK signaling *in vivo*.

2. Materials and methods

2.1. Northern analysis

Tissues from 2-month-old male BALB/c mice were homogenized by grinding in the presence of liquid nitrogen. Total RNA from homogenates was isolated using the TRI reagent (Molecular Research Center), according to the manufacturer's instructions. A probe corresponding to nucleotides 1261–1650 of VRK3 (NCBI accession number BC024839) was generated by PCR amplification, and radioisotope-labeled by random primer extension (Boehringer Mannheim). Probes were purified using a push column beta shield device and NucTrap probe purification columns (Stratagene). An aliquot of total RNA (5 μg) was subjected to Northern blot analysis. Following hybridization, washing and detection procedures were performed, as described previously [26].

2.2. In situ hybridization

The mice were perfused intracardially with 0.1 M phosphate buffered saline (PBS), pH 7.2, followed by a fixative consisting of 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.2. Tissues were cryo-sectioned at up to 4 μm in thickness then mounted onto poly-lysine-coated slides. Tissue sections were deparaffinized, dehydrated, and incubated with either sense or anti-sense riboprobes, prepared as described below.

The probe (nucleotides 1261–1650 of VRK3) for Northern blot analysis was subcloned into pGEM-T Easy vector (Promega), and transcribed by T7 or SP6 RNA polymerase, to generate sense or anti-sense riboprobes, respectively. Riboprobes were purified using a probe purification column (Stratagene), and then used for *in situ* hybridization analysis. Following hybridization, washing and detection procedures were performed as described previously [27].

2.3. Immunohistochemistry

Tissue sections, prepared during *in situ* hybridization, were incubated with an anti-VRK3 or an anti-VHR antibody, followed by incubation with fluorescence-labeled secondary antibodies. DNA was counterstained with 20 ng/ml of Hoechst dye. An Axioplan2 fluorescence imaging microscope (Carl Zeiss), equipped with an ApoTome (Carl Zeiss), was used for obtaining fluorescence or differential interference contrast images.

2.4. Rat embryos

Rat embryos were obtained from Sprague–Dawley rats (Hyochang Science, Daegu, Korea), sacrificed at days 13 to 17, and various organs were collected and analyzed.

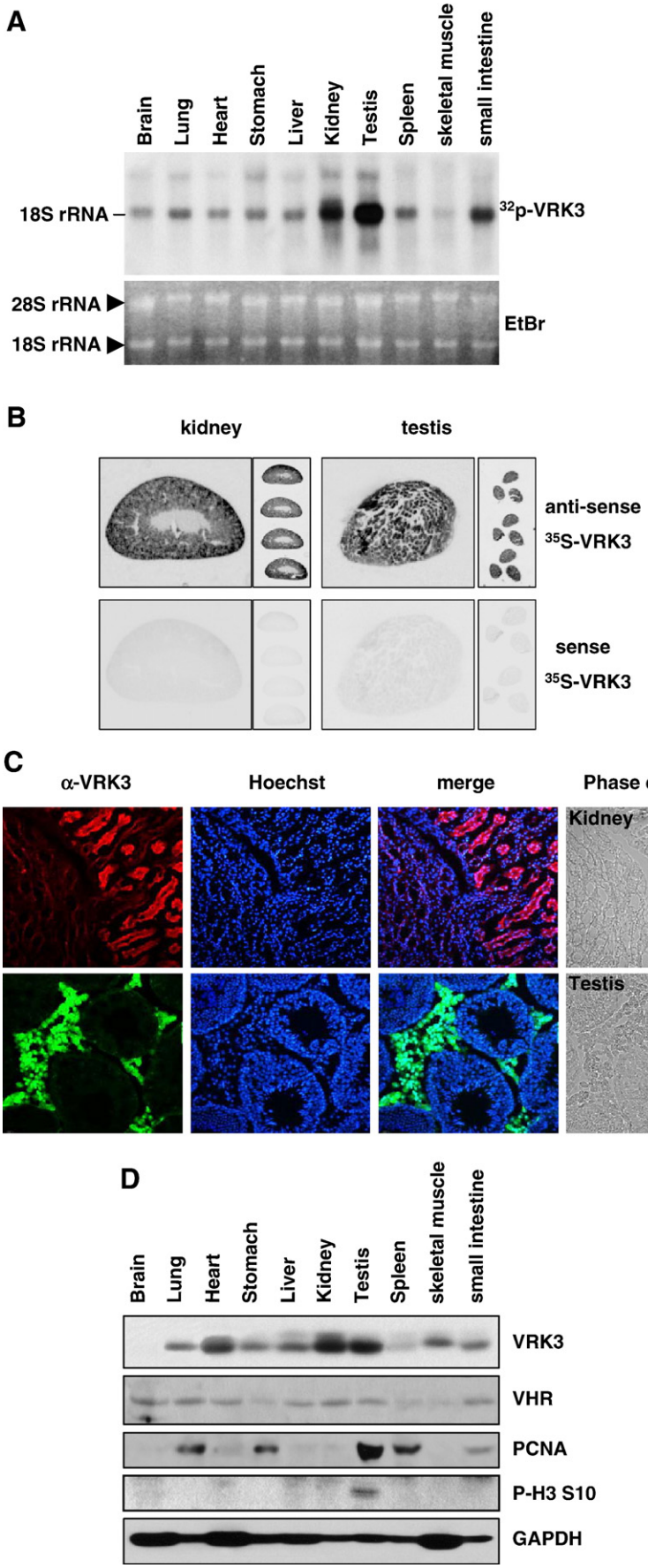
2.5. Immunoblotting

Total protein lysates were prepared in lysis buffer (20 mM HEPES, pH 7.6, 250 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 20% Glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol (DTT), protease inhibitor cocktail (Roche) and 200 μM PMSF), subjected to SDS-PAGE, and transferred to nitrocellulose membranes. Blots were incubated with specific antibodies, including VRK1, VRK2 and VRK3 antisera that were prepared using an established protocol [11]. Other antibodies used were anti-VHR (BD Bioscience), -ERK1/2, -phospho-ERK1/2, -phospho-JNK, -phospho-p38 (Cell Signaling Technology), anti-MKP1, -MKP2 and -PCNA (Santa Cruz Biotechnology), anti-MKP3 (Proteintech Group, Inc), anti-phospho histone H3 Ser10 (Upstate) and anti-glyceraldehyde 3-phosphate dehydrogenase (Calbiochem).

2.6. Immunoprecipitation

For immunoprecipitation, protein lysates from mouse testes were incubated for 2 h at 4 °C with protein-G sepharose (Amersham), and rat preimmune serum or mouse immunoglobulin G1 was used for pre-clearing. The supernatants were transferred to a new tube, and incubated overnight at 4 °C either with rat anti-VRK3 or with mouse anti-VHR antibody. Protein-G sepharose beads were added, incubated for 2 h at 4 °C, washed, and the bound proteins were then

Fig. 1. Tissue distribution of mouse VRK3. (A) Northern blotting with an anti-sense VRK3 probe. Ethidium bromide (EtBr)-stained 18S and 28S ribosomal RNA (rRNA) was used as a loading control. (B) Localization of VRK3 mRNA in kidney and testis was detected with anti-sense (upper panels) or sense VRK3 probes (bottom panels). (C) Localization of VRK3 protein was analyzed by immunohistochemistry with an anti-VRK3 antibody in kidney and testis. VRK3 (red or green), DNA (blue, Hoechst) and DIC (differential interference contrast).


A
Brain
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Testis
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skeletal muscle
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18S rRNA
32p-VRK3
28S rRNA
18S rRNA
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anti-sense
35S-VRK3
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α-VRK3
Hoechst
merge
Phase contrast
Kidney
Testis
D
Brain
Lung
Heart
Stomach
Liver
Kidney
Testis
Spleen
skeletal muscle
small intestine
VRK3
VHR
PCNA
P-H3 S10
GAPDH

eluted with 2× SDS loading buffer. Coimmunoprecipitated proteins were analyzed by immunoblotting with specific antibodies.

2.7. Phosphatase activity assay

VHR or MKP3 proteins were immunoprecipitated from 500 μg of total lysates of tissues or embryos. Immunoprecipitated VHR or MKP3 was then incubated for the indicated period of time (hours) or for 3 h at 30 °C in phosphatase reaction buffer (50 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.5 mM DTT and 150 mM KCl) containing 500 ng of recombinant phosphorylated ERK2 protein as substrate (a gift from Dr. H. S. Yoon, Nanyang Technological University, Singapore). The residual phospho-ERK2 protein level was detected by immunoblotting with an anti-phospho-ERK antibody. The total ERK2 quantity, which served as a loading control, was also measured with a non-phospho anti-ERK antibody.

2.8. Cell culture and transfection

HT22 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml each of penicillin G and streptomycin. Transient transfection of HT22 cells was carried out with Metafectine Reagent (Biontex) as described in the manufacturer's protocol. The siRNA duplex targeting VRK3 (siVRK3; GAGUUCAUUAGCAUGGACC), and the scrambled siRNA (siCont; CTGUUCAAUUACGAGCAGU) were obtained from Dharmacon.

3. Results

3.1. Tissue distribution of mouse VRK3

VRK3 cooperates with VHR to negatively regulate nuclear ERK in resting or nerve growth factor-stimulated neuronal cells [11]. ERK is expressed in almost all cell types, and plays a key role in numerous cellular functions [28]. Accordingly, we wondered whether the interplay between VRK3 and VHR also exists in tissues other than neuronal cells. To test this possibility, we first determined the level of VRK3 and VHR expression in various mouse tissues. The mRNA expression level of VRK3 was initially assessed by Northern blotting. The results revealed abundant levels of VRK3 mRNA expression in the testis and kidney tissue, but very little VRK3 mRNA expression in the skeletal muscle (Fig. 1A). Most other tissues examined displayed moderate VRK3 expression (Fig. 1A). *In situ* hybridization was performed to determine the mRNA localization of VRK3 in the tissues that expressed the highest level of VRK3 (testis and kidney). In the kidney tissue, the VRK3 mRNA signal was preferentially localized to the cortex region, and was hardly detectable in the medulla region (Fig. 1B). Immunohistochemical analysis yielded similar results to *in situ* hybridization and confirmed that cortex-rich expression of VRK3 exists (Fig. 1C). In the testis tissue, VRK3 expression was detected mainly in the interstitial tissues consisting of Leydig cells, which are responsible for the production of male hormones, while in seminiferous tubules, which contain Sertoli and spermatogenic cells, VRK3 was hardly detectable (Fig. 1C). Previous studies have detected relatively high levels of VRK3 during mouse hematopoietic development, indicating that VRK3 expression is associated with cellular proliferation [2]. Of the adult mouse tissues, the testis is known to undergo continuous proliferation. Indeed, the markers of cell proliferation such as phospho-histone H3 and proliferating cell nuclear antigen (PCNA) were prominent in the testis (Fig. 1D). As shown in Fig. 1D, the level of VRK3 was very heterogeneous, while VHR had relatively similar levels in all tissues tested. However, it is striking that the levels of VRK3 in both testis and kidney were very high, but the proliferation markers were only detected in the testis (Fig. 1D). The results suggest that VRK3 level is not directly correlated with cell proliferation. Tissue distribution analyses revealed ubiquitous expression of VRK3, implying its general and various physiological functions *in vivo*.

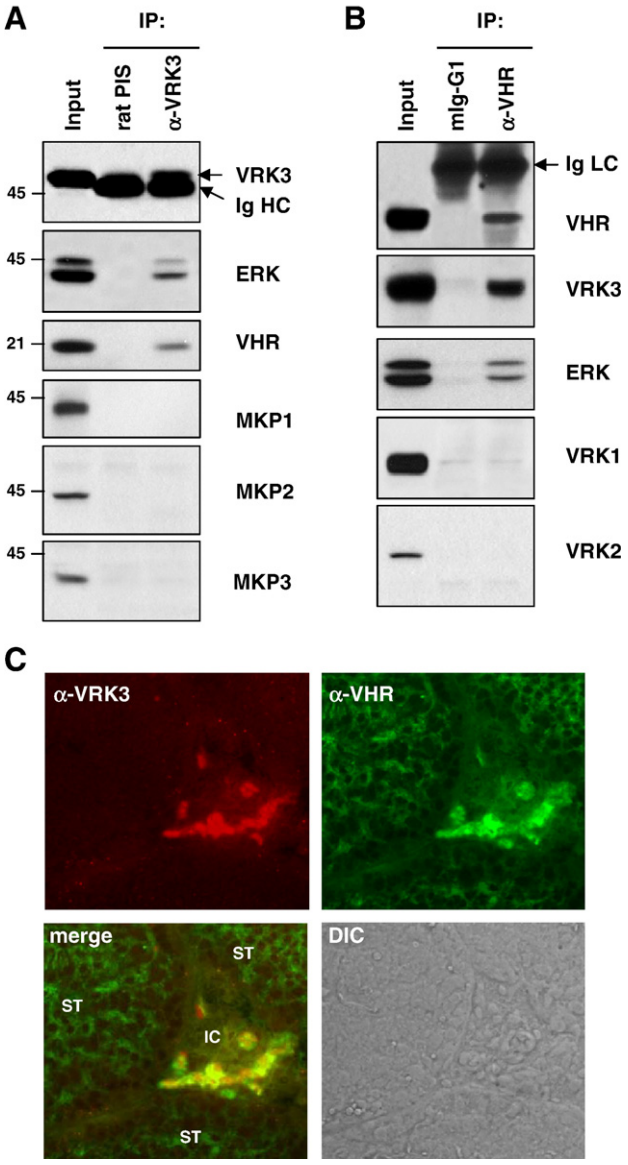

Fig. 2. Ternary complex containing VRK3, VHR, and ERK in mouse testis. Total lysates from mouse testis were analyzed by immunoprecipitation with an anti-VRK3 antibody (A) or with an anti-VHR antibody (B). Coimmunoprecipitated proteins were detected using specific antibodies as indicated. PIS (preimmune serum), mIg-G1 (mouse immunoglobulin G1), Ig HC (immunoglobulin heavy chain) and Ig LC (immunoglobulin light chain). (C) Immunohistochemistry of mouse testis to detect VRK3 (red) and VHR (green). ST; seminiferous tubule, IC; interstitial cells, DIC; differential interference contrast.

3.2. A VRK3/VHR/ERK complex in mouse tissues

In the previous report, we found a ternary complex containing VRK3, VHR and ERK in neuronal cells [11]. To determine whether this protein complex also exists in other tissues besides neuronal cells, we prepared protein lysates from mouse testis and carried out immunoprecipitation analysis. Not surprisingly, VHR and ERK were found in immunoprecipitates prepared with an anti-VRK3 antibody, while other MKPs including MKP1, MKP2 and MKP3 were not detected (Fig. 2A). In turn, when VHR was pulled-down, co-precipitated VRK3 and ERK were detected, whereas other VRK isotypes such as VRK1 and VRK2 were undetectable (Fig. 2B). Immunohistochemical analysis revealed that both VRK3 and VHR were expressed dominantly in the interstitial cells but significant amounts of VHR were also detected in the seminiferous tubules of the testis tissue (Fig. 2C). These results show that VRK3 assembles specifically with VHR in mouse tissues.

3.3. VHR phosphatase activity in mouse tissues

VRK3 mediates ERK inactivation *via* direct binding to VHR phosphatase in a concentration-dependent manner both *in vitro* and *in vivo* [11,29]. To investigate the relationship between VRK3 expression and ERK activity in various mouse tissues, we measured the protein levels of VRK3, VHR and phospho-ERK. Interestingly, the activity of ERK was inversely proportional to VRK3 expression in the tested tissues, whereas a relationship between JNK or p38 MAPK and VRK3 expression was not evident (Fig. 3A). Of importance is the finding that the

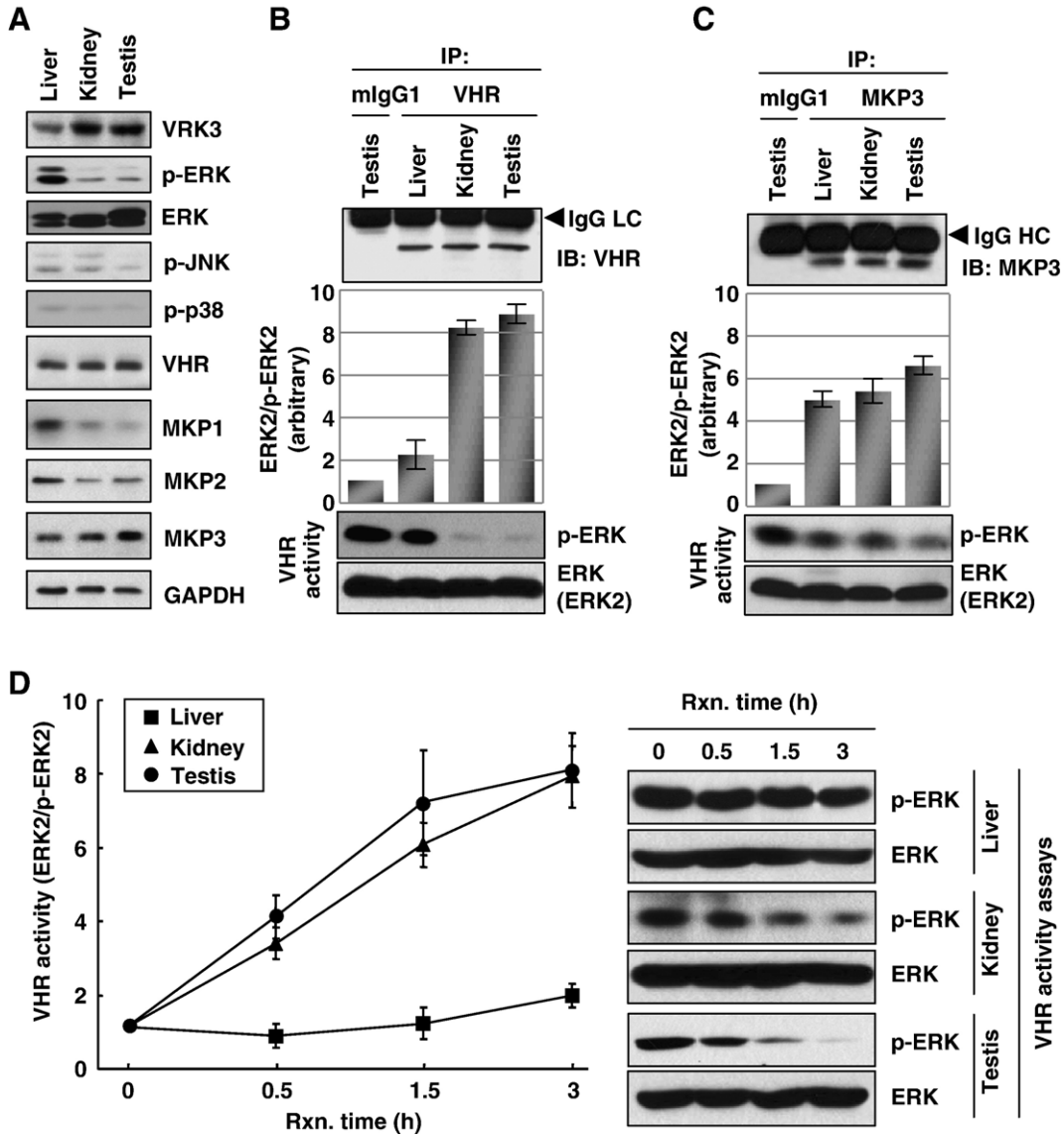

Fig. 3. VRK3, MAPKs and MKPs protein expression and activity assay of constitutively expressed MKPs in mouse tissues. (A) Total lysates from mouse liver, kidney and testis were analyzed by immunoblotting with the indicated antibodies. The level of GAPDH was used to normalize the loading quantity of lysates. (B, C) Immunoprecipitated VHR (B) or MKP3 (C) activity assay from mouse tissues. The ratio of dephosphorylated/phosphorylated ERK2 proteins, used as a substrate, was measured. VHR or MKP3 levels from immunoprecipitates are shown. (D) Phosphatase activity of VHR from different tissues. Error bars in B–D represent means ± S.D. (n = 3).

kidney and testis tissues, which displayed abundant VRK3 protein levels, showed decreased levels of phospho-ERK, whereas the liver, which had lower VRK3 levels, showed elevated levels of active ERK. To determine the MKP responsible for the maintenance of basal phospho-ERK levels in those tissues, we measured the expression levels of MKPs by immunoblotting. The expression of inducible MKPs (MKP1 and MKP2), whose activities are dependent on their expression, was associated with phospho-ERK levels. In contrast, VHR and MKP3 were expressed rather evenly in all three tissues, except for the presence of slightly higher MKP3 levels in the testis (Fig. 3A). These results suggest that inducible MKPs are not responsible for the basal ERK activity in adult mouse tissues. Thus, we shifted our attention to the activity of constitutively expressed MKPs. Immunoprecipitated VHR (Fig. 3B) or MKP3 (Fig. 3C) was used to assess phosphatase activity by measuring the dephosphorylation/phosphorylation ratio of recombinant phospho-ERK2 proteins as substrate. The VHR levels in the precipitates were similar, but the phosphatase activities were distinct among the three tissues tested (Fig. 3B). Specifically, the VHR activity in the kidney and testis (which have abundant VRK3 expression) was more prominent than that observed in the liver (which has low VRK3 expression) (Fig. 3B).

When we investigated the activity of MKP3 in various tissues, we found that the activity of MKP3 differs for each tissue type, but no correlation with the endogenous phospho-ERK levels was observed (Fig. 3C). Given our observation of similar VHR levels but different phosphatase activities in different tissues, we were interested in determining the source of the variations in phosphatase activity. To this end, we measured the VHR phosphatase activity and found that this activity increased in a time-dependent manner in all three tissues tested (Fig. 3D). We further demonstrated that the VHR activity present in the kidney and testis is dramatically increased compared to that observed in the liver. These results show that the intrinsic activity of VHR varies from tissue to tissue, and that this activity is closely correlated with the basal ERK activity in mouse tissues.

3.4. Enhanced VHR activity is mediated by VRK3 in mouse tissues

Given that the activity of ERK is directly affected by VHR activity, and is linked to VRK3 expression, we tested if VRK3 can modulate VHR activity *in vivo*. To confirm this possibility, we prepared purified GST, GST-VRK1, GST-VRK3 or GST-VRK3-KD (kinase-dead) mutant proteins and added each protein to total liver tissue lysates. The effect of adding the purified protein was assessed by measuring the phosphatase activity following immunoprecipitation with VHR. Addition of GST or GST-VRK1 did not alter the VHR activity, whereas GST-VRK3 or GST-VRK3-KD markedly increased VHR activity (Fig. 4A). Moreover, VRK3 increased VHR phosphatase activity in a concentration-dependent manner (Fig. 4B). An *in vitro* binding assay also showed a correlation between VHR activity and the level of associated-VRK3 (Fig. 4C). Thus, these results extend our previous *in vitro* observations and show that

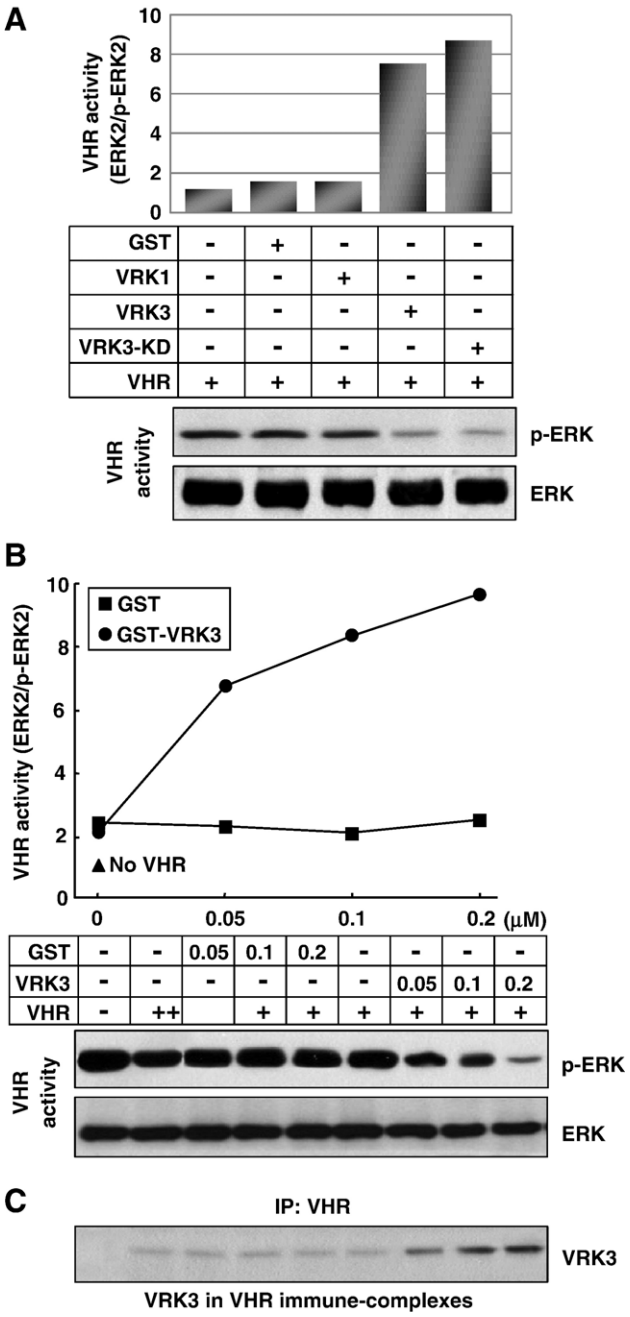

Fig. 4. Essential role of VRK3 in VHR activation. (A) Each recombinant protein (as indicated) was added to the total protein liver extracts for 12 h, VHR phosphatase activity assays were performed *in vitro*, and immunoprecipitation of VHR was performed using a specific antibody against VHR. (B) VHR activity was assessed as in (A) except that increasing concentrations of GST or GST-VRK3 protein were added. (C) The bound-VRK3, obtained in the VHR-immune complex from (B), was detected by immunoblotting with an anti-VRK3 antibody.

VRK3 upregulates VHR phosphatase activity in various other cell systems through a direct interaction.

3.5. Correlation between VRK3 expression and VHR activity during rat embryo development

Next, we examined embryos in order to investigate the involvement of VHR during development, since embryonic tissues

undergo prominent cell growth and differentiation. To assess the expression of VRK3, VHR and ERK in embryos, total protein lysates were prepared from rat embryos at different developmental stages (embryonic days 13 to 17). Interestingly immunoblotting experiments showed that VRK3 and phospho-ERK levels changed between embryonic stages (Fig. 5A). As an embryo matured, the VRK3 expression increased gradually, but the level of phospho-ERK decreased (Fig. 5A). In contrast, the level of VHR protein was similar throughout embryonic maturation, whilst the expression of other MKPs, including MKP1, MKP2 and MKP3, differed between embryonic stages (Fig. 5A). Given the finding that the level of inducible MKPs, such as MKP1 and MKP2, did not show a significant correlation with the level of phospho-ERK, we measured constitutively expressed MKPs such as VHR and MKP3. While the activity of MKP3 was dependent on its expression level (Fig. 5B), the activity of VHR gradually increased despite the fact that the VHR protein level did not vary considerably during embryonic development (Fig. 5B). Thus, of the tested MKPs, the VHR activity seemed to reflect the basal ERK activity in these embryos. To investigate whether VRK3 can modulate VHR activity in this system, similar experiments to those described in Fig. 4B

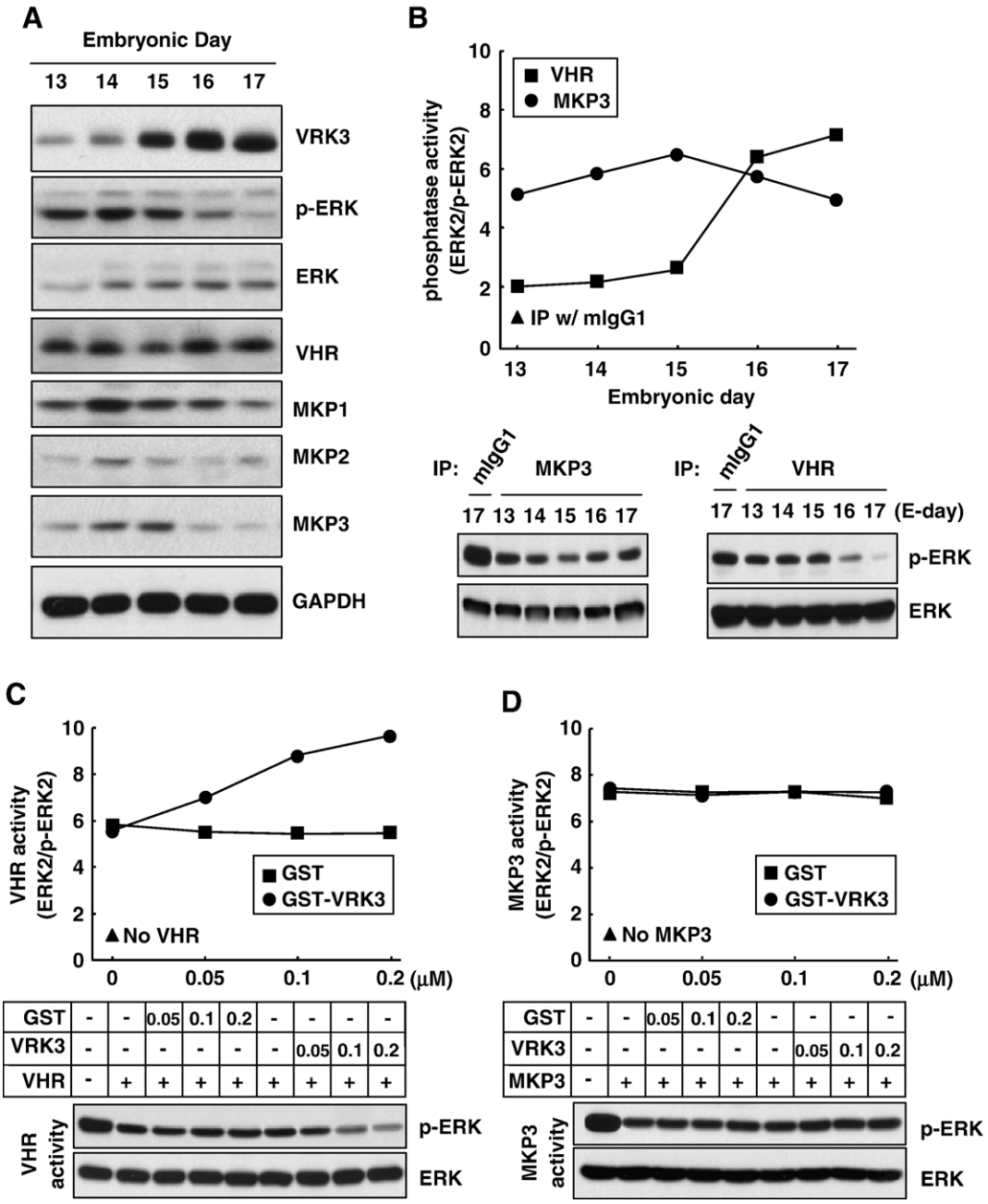

Fig. 5. VRK3 is required for VHR-mediated ERK inactivation during embryo development. (A) Total lysates from rat embryos were analyzed by immunoblotting with the indicated antibodies. (B) Immunoprecipitated MKP3 or VHR phosphatase activity was determined by analyzing the ratio of dephosphorylated/phosphorylated ERK2 protein used as a substrate. (C, D) Modulation of VHR (C) or MKP3 (D) phosphatase activity was assessed by an *in vitro* phosphatase assay containing increasing amounts of GST (control) or GST-VRK3 protein.

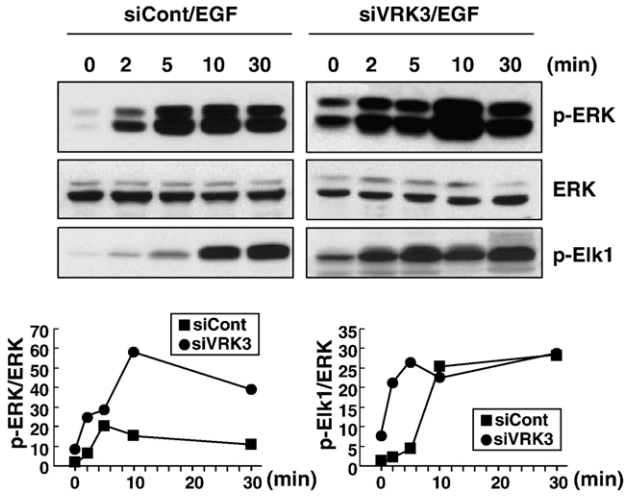

Fig. 6. Potentiation of ERK signaling by knock-down of VRK3. HT22 cells were transfected with either control siRNA or siRNA targeting VRK3 for 24 h and then treated with EGF for the indicated times. Total cell lysates were analyzed by immunoblotting with the indicated antibody. The level of phospho-ERK and phospho-Elk1 was quantitated and depicted.

were conducted. The results show that VHR phosphatase activity is markedly enhanced by the addition of recombinant VRK3 protein in a concentration-dependent manner (Fig. 5C). In contrast, the addition of the GST control failed to activate VHR (Fig. 5C). We further found that MKP3 activity was not altered by the addition of GST-VRK3 protein (Fig. 5D), suggesting that VRK3 can indirectly modulate ERK activity by activating VHR in embryos.

3.6. Potentiation of EGF-induced ERK activity by knock-down of VRK3

Stimulation of cells with epidermal growth factor (EGF) can induce transient activation of ERK within several minutes and induce cell growth [29]. To examine if VRK3 can inhibit the EGF-induced cellular growth signal, we measured ERK activity by measuring the levels of phospho-ERK and phospho-E28 like kinase1 (Elk1). Elk1 is well characterized as a nuclear target of active ERK under growth conditions [30,31,32]. Stimulation of EGF in VRK3-depleted cells resulted in more potent and sustained ERK activation as well as rapid elevation of phospho-Elk1 was detected compared to control siRNA-treated cells (Fig. 6).

4. Discussion

In the present study, we observed abundant VRK3 expression in the renal cortex and Leydig cells of rodent testes. In the renal cortex, the ERK pathway is implicated in numerous diseases such as diabetic renal hypertrophy, diabetic nephropathy, and glomerulosclerosis [33,34,35]. Additionally, ERK has been suggested to play a role in renal development, particularly during glomerulonephrogenesis [36,37]. On the other hand, the ERK pathway is implicated in the production of steroid hormones [38,39] and the regulation of morphological changes in Leydig cells of the testis [40]. Further studies are required to determine the delicate functions of VRK3 in particular tissues and cells, in order to elucidate its precise physiological role in these systems.

We found that the VRK3 protein level directly affected the phosphatase activity of VHR, resulting in reduced ERK activity in both adult tissues and embryos. During embryonic development, VRK3 expression gradually increased whereas ERK activity was decreased progressively. This suggests that VRK3 may play a role in various developmental processes such as proliferation and differentiation by regulating ERK activity. More direct evidence for the role of VRK3 in the activation of VHR *in vivo* could be obtained using VRK3 knock-out mice.

Typical MKPs are composed of two domains: a dual-specificity phosphatase (DUSP) domain and a MAPK-binding (MKB) domain. The DUSP domains of all MKPs share strong homology with each other, while the MKB domain plays a major role in regulating their enzymatic specificity by interacting with MAPKs. The MKB domain contains a cluster of positively charged amino acids, which are involved in determining the binding specificity of MKPs to MAPKs [41,42]. Binding of phosphorylated MAPK to the MKB domain alters the structure of the DUSP domain. This conformational change, along with the interaction of the MKP's catalytic domain with MAPK, greatly enhances the catalytic activity of MKPs [43,44]. The atypical MKP, VHR has a DUSP domain but not a MKB domain [17]. Nevertheless, VHR binds to MAPKs, especially ERK with exquisite specificity [11,21]. As the DUSP domain alone is not enough to selectively distinguish between MAPKs, we speculated that an auxiliary factor imparts MAPK specificity to VHR. From this view point, it is possible that VRK3 functions as the MKB domain of VHR, and thus VRK3 confers its MAPK specificity to VHR. Further studies are required to identify the binding partners of low molecular weight, atypical MKPs, such as MKP6 and MKP8. The role of these factors as MKB domains for specific atypical MKPs will provide important insights into the validity of this hypothesis.

ERK acts as the core protein kinase that links various extracellular stimuli to diverse intracellular physiological responses. Such responses range from gene expression outcomes to decisions made on the fate of a cell [12,13,28]. Constitutive activation of ERK is commonly detected in human tumors, and the protein is therefore an attractive target for anticancer drug development [45,46]. It is speculated that specific inhibition of the ERK pathway blocks abnormal mitogenic signals, metastasis, and angiogenesis in tumors [45,46]. In fact, several inhibitors targeting specific protein kinases in the growth factor-ERK pathway, including RAF and MEK, are currently under development for clinical trials. Here, we propose that VRK3, which activates VHR in a concentration-dependent manner, provides an alternative avenue to alleviate aberrantly upregulated ERK activity. We show here that in both adult and embryonic tissues, VHR significantly contributes to the maintenance of basal ERK activity. VRK3 plays a critical role as a limiting factor of VHR activation since VHR activation is achieved in various tissues by the addition of recombinant VRK3 protein. Hence, regulating ERK, *via* VRK3-mediated

manipulation of VHR activity would be an effective approach to treating various pathological conditions caused by abnormal ERK signaling.

Here, we demonstrate that VRK3 is expressed in various adult tissues and embryos, and that VRK3 enables VHR to play an essential role in negatively affecting ERK activity. VRK3 can therefore be utilized to develop effective modulators of various pathological conditions caused by deregulation of ERK. Collectively, our results indicate that VRK3 is a critical regulator of ERK signaling and confers MAPK specificity to VHR *in vivo*.

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